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(54) Title: DNA ENCODING A HUMAN CALCIUM CHANNEL ALPHA-1E SUBUNIT

(57) Abstract

Isolated DNA encoding a human neuronal calcium channel alpha-1 subunit of subtype E and its corresponding polypeptide are disclosed. Also disclosed are cells expressing the human neuronal calcium channel alpha-1E subunit, as well as methods for screening for therapeutic compounds, using such compositions.

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DNA ENCODING A HUMAN CALCIUM CHANNEL ALPHA-1E SUBUNIT

Field of the Invention

The present invention relates to human calcium channel compositions. In particular, the invention includes compositions containing a human neuronal calcium channel alpha subunit designated subtype "1E" herein. Compositions of the invention include coding sequences for the subunit, as well as cells containing such coding sequences and expressing the human neuronal alpha-1E subunit.

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Background of the Invention

Voltage-gated calcium channels are present in a wide variety of tissues, particularly excitable tissues, where they act to regulate membrane

excitability. Such functions as muscle contraction and synaptic transmission are regulated, at least in part, by specific voltage-gated calcium channels. Compounds, such as dihydropyridine compounds, that block certain of these channels are used therapeutically in the management of various disorders such as hypertension, angina and subarachnoid hemorrhage.

Voltage-gated calcium channels have generally been classified according to their electrophysiological and pharmacological properties. Currently, at least four classes of voltage-gated calcium channels are recognized: L-type, T-type, N-type, and P-type. However, molecular cloning techniques have revealed the existence of different sub-types of channel within some of these classes. (see Tsien et al., Trends in Pharmacological Sciences 12: 349-354, for review).

In general, voltage gated calcium channels have been shown to consist of at least 4 non-identical subunits: the alpha-1 subunit, alpha-2 subunit, beta subunit and gamma subunit. For the L-type calcium channels, which are probably the best characterized calcium channels, it has been shown that the alpha-1 subunit contains the binding site for dihydropyridine ligands.

Partial cDNA clones encoding portions of several different subtypes of of the rat neuronal calcium channel alpha-1 subunit, referred to as rat brain class A, B, C and D, were first isolated from rat brain cDNA libraries (Snutch, *et al.*, 1990). Homologous alpha-1 clones subtypes A-D have been described in humans by Harpold *et al.* (PCT/US92/06903; WO93/04083).

The present invention is concerned with a fifth human neuronal calcium channel alpha-1

subunit, termed "1E" or hα-1E herein. This subunit diverges considerably from the other human alpha-1 subunit clones, exhibiting only about 62% deduced amino acid sequence homology to other human neuronal alpha-1 subunits. As shown herein, this subunit can form a heterologous calcium channel with alpha-2 and beta subunits. The novel channel has a unique pharmacology and is therefore useful in screening for new, more selective therapeutic agents directed at calcium channel modulation.

Summary of the Invention

In one aspect, the invention includes an isolated DNA fragment that encodes an alpha-1E subunit of a human neuronal calcium channel. The fragment has the nucleotide sequence presented as SEQ ID NO:1.

Also included in the invention is an alpha-1E polypeptide subunit of a human neuronal calcium channel having the sequence presented as SEQ ID NO:2. In another aspect, the invention includes a mammalian expression vector containing the nucleotide sequence SEQ ID NO:1.

A eukaryotic cell in acordance with the invention includes a heterologous DNA having the sequence presented as SEQ ID NO:1. The cell expresses a neuronal calcium channel including a polypeptide subunit having the sequence SEQ ID NO: 2. The cells may be further designed to express neuronal calcium channel subunits α_2 and β.

The cell line can be used to screen compounds effective to inhibit calcium uptake by neuronal cells.

These and other objects and features of the invention will be more fully appreciated when the following detailed description of the invention is

read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figure 1 shows the DNA sequence (SEQ ID NO: 1) of the human neuronal calcium channel alpha subunit hα-1E and the deduced amino acid sequence (SEQ ID NO: 2) for the channel subunit; and

Figure 2 shows a tracing of a barium current measured in a eukaryotic cell expressing a heterologous calcium channel including the human neuronal calcium channel alpha subunit hα-1E.

Detailed Description of the Invention

I. Definitions

Unless otherwise indicated, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Current Protocols in Molecular Biology (Ausubel) for definitions and terms of the art.

The terms "heterologous DNA" and "heterologous RNA" refer to nucleotides that are not endogenous to the cell or part of the genome in which they are present; generally such nucleotides have been added to the cell, by transfection, microinjection, electroporation, or the like. Such nucleotides generally include at least one coding sequence, but this coding sequence need not be expressed.

The term "expression vector" refers to vectors that have the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are commercially available. Selection of appropriate expression vectors is within the knowledge of those having skill in the art.

II. Isolation of DNA Coding Sequences for Human Neuronal Alpha-1E Subunit

The diversity of voltage-gated calcium channels between and within tissues as well as across species is becoming more apparent, as new channels having distinct electrophysiological and pharmacological characteristics are reported. The present invention describes a new calcium channel isolated from human brain. As reported herein, this channel is categorized as an alpha subunit of a voltage-gated calcium channel. The novel human channel reported herein is referred to as the human alpha-1E subunit, or hα-1E, in accordance with the nomenclature defined by Snutch for rat brain-derived calcium channel alpha subunits A-D.

A number of overlapping partial cDNA clones were isolated from a human hippocampal library in order to characterize the complete hα-1E coding sequence. The complete hα-1E nucleotide (nt) sequence is depicted in Figure 1 as SEQ ID NO: 1. Also shown is the deduced amino acid sequence (single letter code indicated below nt sequence) as SEQ ID NO: 2. A list of the of four partial cDNA clones used to characterize the α1E sequence and the nucleotide position of each clone relative to the full-length hα-1E sequence (SEQ ID No.1) is shown in Table 1 below.

Table 1

Identification of Partial hα-1E Clones

Name	SEQ ID NO	Location Relative to Full-Length Sequence
H24	3	nt 1 to 2374 of SEQ ID No 1
H6	4	nt 1185 to 5377 of SEQ ID No 1
I2	5	nt 3192 to 6435 of SEQ ID No 1
3-69A	6	nt 5176 to 692[illegible] of SEQ ID No 1

The isolation and characterization of each of the partial clones are described below and detailed in Example 1. The codon for the start methionine begins with nt 107 and nt 6912 represents the end of the open reading frame. The initial 107 and final 10 nucleotides (nt) represent 5′ and 3′ untranslated regions, respectively.

A. Screening Libraries for Human Calcium Channels

Synthetic oligonucleotide probes for hybridization screening were prepared on an automated oligonucleotide synthesizer (Applied Biosystems, Foster City, CA). In order to obtain clones to form the complete hα-1E sequence, oligonucleotide probes based on other calcium channels were constructed as described in Example 1. Probes were constructed based on the sequence of the rat calcium channels such as those described by Snutch and Dubel. These subunits are alpha-1 subunits of rat neuronal calcium channels. They exhibit only about a 62% amino acid sequence identity with the hα-1E clone which was eventually constructed. Further probes were restriction fragments of partial clone H6.

A human hippocampal library was obtained from a commercial supplier (Stratagene, La Jolla, CA). Alternatively, brain libraries from the hippocampal or other discrete brain regions, or from the whole brain, can be produced according to standard methods now known in the art (Ausubel), in order to identify variants, such as splice variants of the hα-1E channel. Such methods include lysing of cells comprising the region of interest to extract RNA. PolyA+ RNA is then isolated and used to synthesize single-stranded cDNA, from which is produced double-stranded cDNA,

using specific or random hexamer primers, such as hexadeoxynucleotide primers (Clontech, Palo Alto, CA).

Restriction enzyme adaptor regions are then ligated to the single-stranded cDNA, according to standard methods (Sambrook). Such cDNA is then purified and size-selected by agarose gel chromatography. The cDNA is then eluted and ligated to a selected vector sequence, such as a lambda gt11 vector or a lambda ZAP vector, as used in the Examples reported herein. The vector is then packaged into appropriate phage hosts and used to infect bacterial cells, as E.coli according to methods known in the art.

Commercial or custom generated libraries, such as phage-cDNA libraries described above, are screened using as probes oligonucleotide hybridization probes, as described in Example 1, under standard conditions, and medium stringency (Ausubel). Briefly, oligonucleotide hybridization probes are radiolabeled by random priming methods (Sambrook), then hybridized to immobilized DNA from the cDNA library. Those phage plaques showing hybridization with the probes are selected, subcloned, and re-tested. Positive clones are identified by autoradiography. Clones identified according to the methods described above are expected to be partial sequence clones, due to the size selection of the cDNA used in generating the library and the predicted size of the calcium channel alpha subunit, based on data from calcium channels from other species.

Clones identified and isolated as described above are placque purified prior to extraction of DNA and production of double stranded plasmid cDNA. The sequence of the plasmid cDNA is then determined, by standard methods, such as on a

commercial DNA sequencer (Applied Biosystems, Foster City, CA). As noted above, four partial clones, H6, H24, I2, and 3-69A, were used to determine the full-length sequence of hα-1E. Regions of overlap between different clones obtained were determined by comparison of the sequences.

Alternatively, the sequences provided by the end-terminal sequences of partial clones useful as specific sequence primers in first-strand DNA synthesis reactions (Maniatis et al.; Scharf et al.) using, for example, partially purified total cellular RNA as substrate. Synthesis of the second-strand of the cDNA is randomly primed (Boehringer Mannheim, Indianapolis IN). The above procedures identify or produce cDNA molecules corresponding to nucleic acid regions that are adjacent to any of the partial clone sequences. These newly isolated sequences can in turn be used to identify further flanking sequences, and so on, to identify overlapping cDNA clones from which the entire hα-1E sequence can be determined. Using the general methods described above, and detailed in Example 1, the sequence of the full-length hα-1E coding sequence is determined. The full-length hα-1E clone is constructed from the partial overlapping clones is detailed in Example 2.

B. Construction of Full-length hα-1E Clones

With reference to Table 1 and Figure 1, it can be seen that α1E cDNA clones H24, H6, I2, and 3-69A, isolated as described above, overlap and include the nucleotide sequence which codes for the entire α1E open reading frame, nt 107 to 6912 (SEQ ID No: 1). Restriction fragments of these partial cDNA clones were ligated together to

generate a full-length α1E cDNA in a eukaryotic expression vector (pcDNA III- Invitrogen). The resulting construct was named NX-HE1.

The construction of hα-1E, termed NX-HE1 and identified as SEQ ID NO: 1, herein, was performed in multiple steps as described in detail in Example 2. Briefly, the H24 clone (SEQ ID NO:3) was recloned into Bluescript SK+ ("BS") (Stratagene, San Diego, CA) to obtain proper orientation within the BS polylinker such that the BS XhoI site was 5′ relative to the coding region, forming Construct 1. An XhoI fragment from Construct 1 was then ligated into the eukaryotic expression vector pcDNA III (Invitrogen, San Diego, CA) to form Construct 2. An XhoI/ApaI fragment from clone H6 (SEQ ID NO: 4) was then ligated into Construct 2 form Construct 3. The remainder of the 3′ end was constructed separately, as follows. An SfiI/BamHI fragment from clone 3-69A was ligated into clone I2 (SEQ ID NO: 5) to form Construct 4. Finally, an ApaI fragment of Construct 4 was ligated into Construct 3 to from the full length clone SEQ ID No.1). This ligated product containing the full length sequence was named NX-HE1.

III. Heterologous Expression of hα-1E in Cells

It can be appreciated that, given the diversity and importance of voltage-gated calcium channels in mammalian physiology, possession of cells which transiently or constitutively express selected channel subtypes, such as the hα-1E subtype calcium channel, would find use in the medical arts, particularly in the areas of diagnosis and/or drug screening. Exemplary assays in these areas are described in Section V.

It can be appreciated that functional expression of calcium currents is particularly useful in practicing parts of the invention described herein. However, expression of a particular heterologous DNA sequence can be monitored to some advantage, using non-functional assays, such as Northern blot assays and protein expression assays, such as immunodetection methods. The present invention provides tools for use in such methods, including oligonucleotide probes and proteins and peptides for production of antibodies for use in immunodetection assays.

A. Preparation of Recombinant Eukaryotic Cells Containing DNA Encoding Heterologous hα-1E Subunits

DNA encoding the hα-1E calcium channel subunit may be introduced into a host cell for expression of the DNA. Methods for introduction of such DNA into cells are known those skilled in the art (Ausubel, Sambrook). Such methods include, for example, transfection of eukaryotic cells with an appropriate expression plasmid vector, such as the vectors described in Section II herein, or a combination of plasmid vectors each containing a different calcium channel subunit, selected from alpha-1, alpha-2, beta, and gamma subunits known in the art to form functional calcium channels (Williams). each encoding one or more distinct genes or with linear DNA, and selection of transfected cells are also well-known in the art (Sambrook, *et al.*, 1989).

Cloned full-length DNA encoding the alpha-1E subunit of a human calcium channel, such as the hα-1E DNA sequence SEQ ID NO: 1, described herein, introduced into a plasmid vector for expression in a eukaryotic cell. Host cells may also be

transfected with linear DNA according to standard methods.

Practice of the present invention can be effectively carried out using any of a number of mammalian expression systems, including yeast cells. However, mammalian expression systems may be preferred for practicing certain aspects of the invention.

Eukaryotic cells suitable for introduction of heterologous hα-1E calcium channel subunit include any cells that are transfectable by such DNA or RNA or into which such DNA may be injected. Preferred host cells are those that can also express the heterologous DNA and RNA. For practicing certain aspects of the invention, such as electrophysiological measurements described in Section IV it is appreciated that it may be desirable that the host cell lack endogenous functionally expressed voltage gated calcium channelse having current characteristics similar to those exhibited by the human alpha-1 calcium channel subunits described herein. In such cases it may be necessary, in order to observe and measure functional expression of the exogenously added alpha-1E subunit, to introduce into the cell heterologous coding sequences encoding the alpha-2 and possibly also the beta calcium channel subunit. As described above, coding sequences for such auxilliary subunits have been published, and expression in vectors suitable for introduction into cells is well within the skill of the practitioner.

In addition, preferred cells for introducing DNA include those that can be transiently or stably transfected and include, but are not limited to, cells of mammalian origin, such as COS cells, mouse L cells, Chinese Hamster Ovary (CHO)

cells, human embryonic kidney (HEK) cells, African green monkey cells, and the like. Preferred cells include DG44 cells and HEK 293 cells, particularly HEK 293 cells that have been adapted for growth in suspension. Additionally *Xenopus laevis* oöcytes find use in the practice of the invention, as described in Part B and Section IV, below. Additionally, yeast cells such as *Saccharomyces cerevisiae* or *Pichia pastoris* may be utilized in practicing the invention.

Heterologous DNA encoding a human alpha-1E subunit, such as the hα-1E calcium channel subunit, may be introduced by any method known to those skilled in the art, such as transfection with a vector containing the DNA sequence that encodes the subunit, such as the DNA sequence SEQ ID NO: 1. Particularly preferred vectors for transfection of mammalian cells are the pSV2dhfr expression vectors, which contain the SV40 early promoter, mouse dhfr gene, SV40 polyadenylation and splice sites and sequences necessary for maintaining the vector in bacteria, cytomegalovirus (CMV) promoter-based vectors such as pCMV or pCDNA1 or PcDNA3 (Invitrogen), and MMTV promoter-based vectors. DNA encoding the human calcium channel subunit hα-1E is been inserted in the vector pcDNA3 such that its expression is regulated by the CMV promoter. Such constructs are suitable for transfecting a number of mammalian cells, including COS cells and HEK 293 cells. Other suitable vectors and cell targets include, but are not limited to pCMV and pREP vectors (obtained from Invitrogen, San Diego, CA).

Stably or transiently transfected mammalian cells may be prepared by methods that are well known in the art. Cells are transfected with an expression vector having a selectable marker gene

such as the gene for thymidine kinase, dihydrofolate reductase, or the like. Stable transfection of cells is conveniently achieved by growing the transfected cells under conditions that promote growth of cells expressing the marker gene.

The heterologous DNA encoding the human alpha-1E calcium channel subunit may be integrated into cell's chromosomal material or may be maintained in the cell as an episomal element. Cells containing such heterologous DNA may be passaged and/or subcultured, according to methods appropriate to the cell type and known in the art.

B. Functional Expression of hα-1E in Xenopus oocytes

A plasmid, such as a pBluescript SK+ plasmid, containing the full-length NX-HE1 coding sequence is used to produce complementary RNA, using an appropriate RNA polymerase, such as T7 or SP6 polymerase. Such RNA is injected into oocytes from *Xenopus laevis* (about 5-10 pg/oocyte), according to methods known in the art. For expression of a funtional calcium channel in oocytes, coexpression of a calcium channel alpha-2 subunit, such as the rabbit skeletal muscle alpha-2 subunit (Mori et al., incorporated herein by reference) may be required. Additionally, inclusion of a calcium channel Beta subunit, such as the Beta-3 subunit from rabbit heart (Hulin et al, incorporated herein by reference), helps optimize functional expression of the channel. Coding sequences for the aforementioned alpha-2 and beta-3 subunits have been published as cited above, and construction of expression vectors suitable for injection into oocytes is within the skill of the practitioner. For expression in

oocytes, approximately equimolar amounts of RNA are injected into each oocyte.

C. Functional Expression of hα-1E in Mammalian Cells

One cell type that is particularly amenable to transient or stable transfection is the human embryonic kidney cell line HEK 293 (ATCC Accession No. CRL1573). Such cells can be transiently cotransfected with the hα-1E subunit cDNA expression plasmid, and one or more of an α_2 calcium channel subunit cDNA expression plasmid, an β_1 subunit cDNA expression plasmid as detailed in Example 4 (Williams).

Stable transfection can also be achieved in HEK 293 cells according to standard methods (Ausubel). Suitable vectors for stable transfection include pcDNA1 and pcDNA3.

Transfected cells are selected, for example by differential growth in limiting medium, according to methods known in the art. Such cells are subcloned and tested, for example by Northern blot analysis, for the evidence for the expression of the human alpha-1E calcium channel. Alternatively or in addition, individual transfected cells can be analyzed electrophysiologically for the presence of voltage-activated calcium currents (using barium as carrier, as described in Section IV. Such cells are useful in functional and/or binding assays, as described in Section V.

IV. Electrophysiological Measurements

A. Recording of Calcium currents in *Xenopus* oocytes

Functional expression of a heterologous hα-1E calcium channel subunit can be measured in *Xenopus* oocytes injected with heterologous RNA, as

described in Section III. Currents are conveniently recorded using a standard two-microelectrode voltage-clamp connected to an amplification system. Cells are placed in a chloride-free bathing solution containing about 40 mM $Ba(OH)_2$, such that barium acts as current carrier in the system. Channels are activated by periodic delivery of voltage pulses. Currents are measured and normalized according to procedures known to those familiar with the art pertaining to electrophysiology.

V. Utility

The present invention includes methods for identifying therapeutic compounds, such as calcium channel agonist and antagonists, that modulate the activity of calcium channels. Such assays are useful as screens for new therapeutic compounds having calcium channel agonist and/or antagonist activity. Presently, calcium channel antagonists directed at L-type calcium channels find use in the treatment of hypertension, subarachnoid hemorrhage and variant forms of angina. Certain N-type calcium channel antagonists have been found to be useful in reducing cerebral ischemia, as described in co-owned U.S. Patents 4,051,403 and 5,189,020, incorporated herein by reference.

According to the present invention, eukaryotic cells expressing heterologous hα-1E calcium channel subunits encoded by heterologous DNA as described herein are useful for screening for hα-1E subtype-specific compounds, and for predicting the relative potencies of such compounds.

In particular, cells expressing such heterologous calcium channels of the hα-1E subtype can be used in binding assays, wherein whole cells

or membranes thereof are tested for ability to bind a test compound, generally by measuring the ability of the test compound to displace a known ligand of the channel. One appropriate functional ligand which binds to the channel and blocks hα-1E calcium currents is the spider toxin Aga-IIIA (isolated as described by Mintz, et al., incorporated herein by reference).

Methods for carrying out such screening assays are well known in the art, and setting up such assays is within the skill of the practioner. Co-owned allowed U.S. patent application 07/855,269, incorporated herein by reference, describes assays which utilize isolated neuronal calcium channels for screening of certain types of N-type calcium channel antagonists. Such methods are adaptable to the present invention.

Alternatively, or additionally, such screening assays may include assays in which is determined the functional activity of an expressed calcium channel. In such an assay, drugs which alter such activity, such as calcium current activity, are candidates for calcium channel-based therapeutics, of the types suggested above or or additional types.

The following examples illustrate, but in no way are intended to limit the present invention.

Materials and Methods

A lambda-ZAP cDNA human hippocampal cDNA library was obteined from Stratagene (La Jolla, CA). A eukaryotic expression vector pcDNA III was obtained from Invitrogen (San Diego CA). T4 DNA ligase and T4 DNA polymerase were obtained from New England Biolabs (Beverly, MA); Nitrocellulose

filters were obtained from Schleicher and Schuell (Keene, NH).

Bluescript SK+(BS) was obtained from Stratagene (San Diego, CA).

Dephosphorylated Calf intestinal phosphatase (CIP) was obtained form New England Biolabs (Beverly, MA).

Synthetic oligonucleotide linkers and primers were prepared using commercially available automated oligonucleotide synthesizers, such as obtained from Applied Biosystems (Foster City, CA). Alternatively, custom designed synthetic oligonucleotides may be purchased, for example, from Synthetic Genetics (San Diego, CA). cDNA synthesis kit and random priming labeling kits were obtained from Boehringer-Mannheim Biochemical (BMB, Indianapolis, IN).

Standard molecular biology and cloning techniques were performed essentially as previously described in Ausubel, *et al.*, Sambrook, *et al.*, and Maniatis, *et al.*

Example 1

Isolation of partial cDNA clones

A. Clone H6

One million recombinants of a λZAP II (Stratagene, La Jolla, CA) human hippocampal cDNA library were screened in duplicate at a density of 50,000 plaques per 150 mm plate using two radiolabeled 1.6 kb (Hind III and Xho I digested) fragments of the rat Class B α1 subunit cDNA (for the sequence of the rat Class B α1 subunit see Dubel *et al.* (1992) Proc. Natl. Acad. Sci. 89:5058-5062, incorporated herein by reference):

Fragment	Nucleotides
HindIII-HindIII	712 to 2288
Xhol-Xhol	3793-5394

The hybridization was performed under standard conditions (5x SSPE, 5X Denhardt's, 0.1% SDS, 1 mg/ml salmon sperm DNA; recipes found in Sambrook *et al.*(1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press). Filters were washed under medium stringency conditions (0.2X SSPE, 0.1% SDS, 50°C). H6 was one of two Class E specific clones isolated. H6 bacteriophage was plaque purified using standard methods (J. Sambrook *et al.* (1989) Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press), double stranded BS plasmid cDNA was isolated using standard phagemid rescue procedures (Stratagene), and DNA sequence was obtained using PCR based fluorescence dye termintaor procedures (Applied Biosystems).

B. Clone H24

One million recombinants of a λZAP II (Stratagene) human hippocampal cDNA library were screened in duplicate at a density of 50,000 plaques per 150 mm plate using a radiolabeled NotI/EcoRI fragment (nucleotides 118 to 705) of the rat Class B α1 subunit cDNA (for the sequence of the rat Class B α1 subunit see Dubel *et al.* (1992) Proc. Natl. Acad. Sci. 89:5058-5062). The hybridization was performed under standard conditions. Filters were washed under medium stringency conditions H24 was one of 5 positives isolated in the screen (4 of which were Class E clones). H24 bacteriophage was plaque purified,

double standard plasmid cDNA was prepared, and the clone was chartacterized by DNA sequencing.

C. Clone I2

One million recombinants of a λZAP II (Stratagene) human hippocampal cDNA library were screened in duplicate at a density of 50,000 plaques per 150 mm plate using a radiolabeled PstI/ ApaI fragment (nucleotides 4194 to 4740) from the cDNA fragment H6. The hybridization was performed under standard conditions. Filters were washed under medium stringency conditions. I2 was one of 13 positives isolated in the screen. I2 bacteriophage was plaque purified, double standard plasmid cDNA was prepared, and the clone was chartacterized by DNA sequencing.

D. Clone 3-69A

One million recombinants of a λZAP II (Stratagene) human hippocampal cDNA library were screened in duplicate at a density of 50,000 plaques per 150 mm plate using a radiolabeled DNA oligonucleotide probe derived from a rat neuronal alpha subunit cDNA. The hybridization was performed under standard conditions. Filters were washed under medium stringency conditions. 3-69A was one of 37 positives isolated in the screen. 3-69A bacteriophage was plaque purified, double standard plasmid cDNA was prepared, and the clone was chartacterized by DNA sequencing.

Example 2

Construction of full length Human α1E cDNA

hα-1E cDNA clones H24, H6, I2, and 3-69A overlap and include the nucleotide sequence which codes for the entire hα-1E open reading frame, nt 107 to 6912 (SEQ ID No: 1). Restriction

fragments of these partial cDNA clones were ligated together to generate a full-length hα-1E cDNA in a eukaryotic expression vector (pcDNA III-Invitrogen). The resulting construct was named NX-HE1. The construction of NX-HE1 was performed in multiple steps as described in detail below: 1) recloning of H24 into Bluescript SK+(BS) to obtain proper orientation (Construct 1), 2) ligation of an XhoI fragment (nt 1 to 1374) from Construct 1 into pcDNA III (to form Construct 2), 3) ligation of an XhoI/ApaI fragment from H6 (nt 1374 to 4181) into Construct 2 (to form Construct 3), 4) ligation of an SfiI/BamHI fragment from 3-69A (nt 5884 to 3′ untranslated) into I2 (to form Construct 4), and 5) ligation of an ApaI fragment (nt 4181 to 3′ untranslated into Construct 3 to from the full length clone SEQ ID No.1).

1) To obtain Construct 1, H24 was digested with EcoRI and religated into EcoRI digested BS. The purpose of this ligation was to reorient the original H24 clone within the BS polylinker such that the BS XhoI site was 5′ relative to the coding region.

2) Construct 2 was digested with XhoI and the resultant 1.4 kb fragment was purified from an agarose gel and ligated into XhoI digested pcDNAIII dephosphorylated with calf intestinal phosphatase (CIP). This construct thus contained the 5′ end of the clone (nt 1 to 1374).

3) H6 was then digested with XhoI and Apa I and the resultant 2.8 kb fragment (nt 1374 to 4181) was ligated into XhoI /ApaI digested and CIP treated Construct 2. The ligated product was referred to as Construct 3.

4) The remainder of the 3′ end was constructed separately. First, 3-69A was digested with SfiI and BamHI to yield a 2.7 kb fragment (nt

5884 to 3' untranslated), which was gel purified and ligated into SfiI/BamHI digested and CIP treated I2. The ligated product was referred to as Construct 4. (All ligation junctions were sequenced prior to proceeding with the next step).

5) The final construct was prepared by digesting Construct 4 with ApaI, yielding a 4.1 kb fragment (nt 4182 to 3'ut) which was gel purified and ligated into ApaI cut CIP treated Construct 3. This ligated product containing the full length sequence was named NX-HE1.

Example 3

Functional Expression of hα-1E in Xenopus oocytes

The hα-1E full-length DNA sequence SEQ ID NO:1 was subcloned into pBluescript SK + to obtain plasmid phα-1E(SK+). Complementary RNA was synthesized from the plasmid *in vitro* using T7 or SP6 polymerase. Additionally, complementary RNA is synthesized from plasmids containing calcium channel alpha-2 subunits and Beta subunits known in the art (Mori, Williams, Tanabe). RNA from each subunit was injected in equimolar ratios using 0.1 $\mu g\ ml^{-1}$ α_1, 0.1 $\mu g\ ml^{-1}$ α_2, and 0.03 $\mu g\ ml^{-1}$ β; ~ 50 nl is injected per cell. The hα-1E currents were recorded by a standard two-microelectrode voltage-clamp using a Warner amplifier (OC-725A) in a chloride free bath solution, containing (in mM): $Ba(OH)_2$, 5; tetraethylammonium-OH, 40; KOH, 2; HEPES, 5; pH 7.4 adjusted with methanesulphonic acid. Data were sampled at 10 kHz and filtered at 2 kHz. Leak and capacitance currents are subtracted off-line by a P/4 protocol. Voltage pulses are delivered every 15 s. For steady-state inactivation experiments, voltage pulses were delivered every 20 s. Peak normalized currents

were fitted to a Hodgkin-Huxley inactivation curve. Figure 2 shows a trace of a barium current measured in a Xenopus oocyte previously injected with mRNA encoding the human calcium channel subunit hα-1E (SEQ ID NO:2) plus RNA encoding alpha-2 and beta subunits, as described above. The current was invoked from a holding potential of -80 mV to a test potential of OmV.

The spider toxin AgaIIIA was effective to block this current, at a batch concentration of 50 nM. Other calcium channel blocking compounds which were unable to block this current and maximum concentrations tested are as follows: omega conotoxins MVIIA (5 μM), MVIIC (5 μM), TVIA (5 μM), Aga IVA (100 nM) and dehydropyridines.

Example 4

Expression of hα-1E in Mammalian Cells

Human embryonic kidney cells (HEK 293 cells) were transiently and stably transfected with human neuronal DNA encoding calcium channel subunits.

A. Transfection of HEK 293 Cells

Separate expression vectors containing DNA encoding human neuronal calcium channel hα-1E, rat α_2, and β_1 subunits, were used. constructed as described in Example 2. The hα-1E coding sequence described herein as SEQ ID NO: 1 was incorporated into a pcDNA1+3 vector, as described above, with addition of a c-myc epitope tag on the 5′ end of the hα-1E coding sequence, for monitoring, according to established methods. The vector was used for stable transfection of HEK 293 cells using the calcium phosphate transfection procedure (Ausubel). Culture plates containing about two million HEK 293 cells, were transfected with 1 ml

of DNA/calcium phosphate precipitate containing 5 μg of each of the vectors. After 10-20 days of growth in media containing 500 μg G418, colonies had formed and were isolated according to standard procedures.

Expression of hα-1E subunit by cells was monitored by fluorescence immunolabeling. Cells were incubated with fluorscently tagged antibodies (from commercial sources) directed to the c-myc epitope, the coding sequence for which was added to the 5′ end of the hα-1E coding sequence. Expression of the hα-1E subunit having the epitope was evidenced by immunofluorescence.

While the invention has been described with reference to specific methods and embodiments, it will be appreciated that various modifications and changes may be made without departing from the invention.

IT IS CLAIMED:

1. An isolated DNA fragment, comprising a sequence of nucleotides that encodes an alpha-1E subunit of a human neuronal calcium channel, and having the sequence presented as SEQ ID NO:1.

2. An alpha-1E polypeptide subunit of a human neuronal calcium channel having the sequence presented as SEQ ID NO:2.

3. A mammalian expression vector containing the nucleotide sequence presented as SEQ ID NO:1.

4. A eukaryotic cell, which includes a heterologous DNA having the sequence presented as SEQ ID NO:1.

5. The cell of claim 4, wherein the cell is a Xenopus oocyte.

6. The cell of claim 4, wherein the cell is a human embryonic kidney cell.

7. The cell of claim 4, wherein said DNA expresses a neuronal calcium channel including a polypeptide subunit having the sequence presented as SEQ ID NO: 2.

8. The cell line of claim 7, which further expresses neuronal calcium channel subunits α_2 and β.

9. A method of screening a compound capable of blocking calcium uptake in human neuronal cells, comprising

exposing the cell of claim 7 with the test compound,

examining the effect of said exposing on calcium uptake into the cells, and

selecting the compound if said exposing inhibits calcium uptake into the cells.

10. The method of claim 9, wherein said exposing includes contacting the cells with the cell of claim 8.

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  1  GAA TTC CGG CTC TGA GTC TCC GTG TGT CTT TCT GCT TGT TGC TGT GTG    48
  1   E   F   R   L   *   V   S   V   C   L   S   A   C   C   C   V    16

 49  CGG GTG TTC GGC CGC GAT CAC CTT TGT GTG TCT TCT GTC TGT TTA AAC    96
 17   R   V   F   G   R   D   H   L   C   V   S   S   V   C   L   N    32

 97  CTC AGG ATG GCT CGC TTC GGG GAG GCG GTG GTC GCC AGG CCA GGG TCC   144
 33   L   R   M   A   R   F   G   E   A   V   V   A   R   P   G   S    48

145  GGC GAT GGA GAC TCG GAC CAG AGC AGG AAC CGG CAA GGA ACC CCC GTG   192
 49   G   D   G   D   S   D   Q   S   R   N   R   Q   G   T   P   V    64

193  CCG GCC TCG GGG CAG GCG GCC GCC TAC AAG CAG ACG AAA GCA CAG AGG   240
 65   P   A   S   G   Q   A   A   A   Y   K   Q   T   K   A   Q   R    80

241  GCG CGG ACT ATG GCT TTG TAC AAC CCC ATT CCC GTC CGG CAG AAC TGT   288
 81   A   R   T   M   A   L   Y   N   P   I   P   V   R   Q   N   C    96

289  TTC ACC GTC AAC AGA TCC CTG TTC ATC TTC GGA GAA GAT AAC ATT GTC   336
 97   F   T   V   N   R   S   L   F   I   F   G   E   D   N   I   V   112

337  AGG AAA TAT GCC AAG AAG CTC ATC GAT TGG CCG CCA TTT GAG TAC ATG   384
113   R   K   Y   A   K   K   L   I   D   W   P   P   F   E   Y   M   128


385  ATC CTG GCC ACC ATC ATT GCC AAC TGC ATC GTC CTG GCC CTG GAG CAG   432
129   I   L   A   T   I   I   A   N   C   I   V   L   A   L   E   Q   144

433  CAT CTT CCT GAG GAT GAC AAG ACC CCC ATG TCC CGA AGA CTG GAG AAG   480
145   H   L   P   E   D   D   K   T   P   M   S   R   R   L   E   K   160

481  ACA GAA CCT TAT TTC ATT GGG ATC TTT TGC TTT GAA GCT GGG ATC AAA   528
161   T   E   P   Y   F   I   G   I   F   C   F   E   A   G   I   K   176

529  ATT GTG GCC CTG GGG TTC ATC TTC CAT AAG GGC TCT TAC CTC CGC AAT   576
177   I   V   A   L   G   F   I   F   H   K   G   S   Y   L   R   N   192
```

Fig. 1

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577  GGC TGG AAT GTC ATG GAC TTC ATC GTG GTC CTC AGT GGC ATC CTG GCC   624
193   G   W   N   V   M   D   F   I   V   V   L   S   G   I   L   A   208

625  ACT GCA GGA ACC CAC TTC AAT ACT CAC GTG GAC CTG AGG ACC CTC CGG   672
209   T   A   G   T   H   F   N   T   H   V   D   L   R   T   L   R   224

673  GCT GTG CGT GTC CTG CGG CCT TTG AAG CTC GTG TCA GGG ATA CCT AGC   720
225   A   V   R   V   L   R   P   L   K   L   V   S   G   I   P   S   240

721  CTG CAG ATT GTG TTG AAG TCC ATC ATG AAG GCC ATG GTA CCT CTT CTG   768
241   L   Q   I   V   L   K   S   I   M   K   A   M   V   P   L   L   256

769  CAG ATT GGC CTT CTG CTC TTC TTT GCC ATC CTG ATG TTT GCT ATC ATT   816
257   Q   I   G   L   L   L   F   F   A   I   L   M   F   A   I   I   272

817  GGT TTG GAG TTC TAC AGT GGC AAG TTA CAT CGA GCG TGC TTC ATG AAC   864
273   G   L   E   F   Y   S   G   K   L   H   R   A   C   F   M   N   288

865  AAT TCA GGT ATT CTA GAA GGA TTT GAC CCC CCT CAC CCA TGT GGT GTG   912
289   N   S   G   I   L   E   G   F   D   P   P   H   P   C   G   V   304

913  CAG GGC TGC CCA GCT GGT TAT GAA TGC AAG GAC TGG ATC GGC CCC AAT   960
305   Q   G   C   P   A   G   Y   E   C   K   D   W   I   G   P   N   320

961  GAT GGG ATC ACC CAG TTT GAT AAC ATC CTT TTT GCT GTG CTG ACT GTC  1008
321   D   G   I   T   Q   F   D   N   I   L   F   A   V   L   T   V   336

1009 TTC CAG TGC ATC ACC ATG GAA GGG TGG ACC ACT GTG CTG TAC AAT ACC  1056
337   F   Q   C   I   T   M   E   G   W   T   T   V   L   Y   N   T   352

1057 AAT GAT GCC TTA GGA GCC ACC TGG AAT TGG CTG TAC TTC ATC CCC CTC  1104
353   N   D   A   L   G   A   T   W   N   W   L   Y   F   I   P   L   368

1105 ATC ATC ATT GGA TCC TTC TTT GTT CTC AAC CTA GTC CTG GGA CTG CTT  1152
369   I   I   I   G   S   F   F   V   L   N   L   V   L   G   V   L   384

1153 TCC GGG GAA TTT GCC AAA GAG AGA GAG AGA GTG GAG AAC CGA AGG GCT  1200
385   S   G   E   F   A   K   E   R   E   R   V   E   N   R   R   A   400
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Fig. 1 (1 con't)

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1201  TTC ATG AAG CTG CGG CGC CAG CAG CAG ATT GAG CGT GAG CTG AAT GGC  1248
 401   F   M   K   L   R   R   Q   Q   Q   I   E   R   E   L   N   G   416

1249  TAC CGT GCC TGG ATA GAC AAA GCA GAG GAA GTC ATG CTC GCT GAA GAA  1296
 417   Y   R   A   W   I   D   K   A   E   E   V   M   L   A   E   E   432

1297  AAT AAA AAT GCT GGA ACA TCC GCC TTA GAA GTG CTT CGA AGG GCA ACC  1344
 433   N   K   N   A   G   T   S   A   L   E   V   L   R   R   A   T   448

1345  ATC AAG AGG AGC CGG ACA GAG GCC ATG ACT CGA GAC TCC AGT GAT GAG  1392
 449   I   K   R   S   R   T   E   A   M   T   R   D   S   S   D   E   464

1393  CAC TGT GTT GAT ATC TCC TCT GTG GGC ACA CCT CTG GCC CGA GCC AGT  1440
 465   H   C   V   D   I   S   S   V   G   T   P   L   A   R   A   S   480

1441  ATC AAA AGT GCA AAG GTA GAC GGG GTC TCT TAT TTC CGG CAC AAG GAA  1488
 481   I   K   S   A   K   V   D   G   V   S   Y   F   R   H   K   E   496

1489  AGG CTT CTG CGC ATC TCC ATT CGC CAC ATG GTT AAA TCC CAG GTG TTT  1536
 497   R   L   L   R   I   S   I   R   H   M   V   K   S   Q   V   F   512

1537  TAC TGG ATT GTG CTG AGC CTT GTG GCA CTC AAC ACT GCC TGT GTG GCC  1584
 513   Y   W   I   V   L   S   L   V   A   L   N   T   A   C   V   A   528

1585  ATT GTC CAT CAC AAC CAG CCC CAG TGG CTC ACC CAC CTC CTC TAC TAT  1632
 529   I   V   H   H   N   Q   P   Q   W   L   T   H   L   L   Y   Y   544

1633  GCA GAA TTT CTG TTT CTG GGA CTC TTC CTC TTG GAG ATG TCC CTG AAG  1680
 545   A   E   F   L   F   L   G   L   F   L   L   E   M   S   L   K   560

1681  ATG TAT GGC ATG GGG CCT CGC CTT TAT TTT CAC TCT TCA TTC AAC TGC  1728
 561   M   Y   G   M   G   P   R   L   Y   F   H   S   S   F   N   C   576

1729  TTT GAT TTT GGG GTC ACA GTG GGC AGT ATC TTT GAA GTG GTC TGG GCA  1776
 577   F   D   F   G   V   T   V   G   S   I   F   E   V   V   W   A   592

1777  ATC TTC AGA CCT GGT ACG TCT TTT GGA ATC AGT GTC TTG CGA GCC CTC  1824
 593   I   F   R   P   G   T   S   F   G   I   S   V   L   R   A   L   608
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Fig. 1 (2 con't)

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1825  CGG CTT CTA AGA ATA TTT AAA ATA ACC AAG TAT TGG GCT TCC CTA CGG  1872
 609    R   L   L   R   I   F   K   I   T   K   Y   W   A   S   L   R   624

1873  AAT TTG GTG GTC TCC TTG ATG AGC TCA ATG AAG TCT ATC ATC AGT TTG  1920
 625    N   L   V   V   S   L   M   S   S   M   K   S   I   I   S   L   640

1921  CTT TTC CTC CTC TTC CTC TTC ATC GTT GTC TTT GCT CTC CTA GGA ATG  1968
 641    L   F   L   L   F   L   F   I   V   V   F   A   L   L   G   M   656

1969  CAG TTA TTT GGA GGC AGG TTT AAC TTT AAT GAT GGG ACT CCT TCG GCA  2016
 657    Q   L   F   G   G   R   F   N   F   N   D   G   T   P   S   A   672

2017  AAT TTT GAT ACC TTC CCT GCA GCC ATC ATG ACT GTG TTC CAG ATC CTG  2064
 673    N   F   D   T   F   P   A   A   I   M   T   V   F   Q   I   L   688

2065  ACG GGT GAG GAC TGG AAT GAG GTG ATG TAC AAT GGG ATC CGC TCC CAG  2112
 689    T   G   E   D   W   N   E   V   M   Y   N   G   I   R   S   Q   704

2113  GGT GGG GTC AGC TCA GGC ATG TGG TCT GCC ATC TAC TTC ATT GTG CTC  2160
 705    G   G   V   S   S   G   M   W   S   A   I   Y   F   I   V   L   720

2161  ACC TTG TTT GGC AAC TAC ACG CTA CTG AAT GTG TTC TTG GCT ATC GCT  2208
 721    T   L   F   G   N   Y   T   L   L   N   V   F   L   A   I   A   736

2209  GTG GAT AAT CTC GCC AAC GCC CAG GAA CTG ACC AAG GAT GAA CAG GAG  2256
 737    V   D   N   L   A   N   A   Q   E   L   T   K   D   E   Q   E   752

2257  GAA GAA GAG GCC TTC AAC CAG AAA CAT GCA CTG CAG AAG GCC AAG GAG  2304
 753    E   E   E   A   F   N   Q   K   H   A   L   Q   K   A   K   E   768

2305  GTC AGC CCG ATG TCT GCA CCC AAC ATG CCT TCG ATC GAA AGA GAC AGA  2352
 769    V   S   P   M   S   A   P   N   M   P   S   I   E   R   D   R   784

2353  AGG AGA AGA CAC CAC ATG TCG ATG TGG GAG CCA CGC AGC AGC CAC CTG  2400
 785    R   R   R   H   H   M   S   M   W   E   P   R   S   S   H   L   800
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Fig. 1 (3 con't)

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2401  AGG GAG CGG AGG CGC CGG CAC CAC ATG TCC GTG TGG GAG CAG CGT ACC   2448
 801    R   E   R   R   R   R   H   H   M   S   V   W   E   Q   R   T    816

2449  AGC CAG CTG AGG AAG CAC ATG CAG ATG TCC AGC CAG GAG GCC CTC AAC   2496
 817    S   Q   L   R   K   H   M   Q   M   S   S   Q   E   A   L   N    832

2497  AGA GAG GAG GCG CCG ACC ATG AAC CCG CTC AAC CCC CTC AAC CCG CTC   2544
 833    R   E   E   A   P   T   M   N   P   L   N   P   L   N   P   L    848

2545  AGC TCC CTC AAC CCG CTC AAT GCC CAC CCC AGC CTT TAT CGG CGA CCC   2592
 849    S   S   L   N   P   L   N   A   H   P   S   L   Y   R   R   P    864

2593  AGG GCC ATT GAG GGC CTG GCC CTG GGC CTG GCC CTG GAG AAG TTC GAG   2640
 865    R   A   I   E   G   L   A   L   G   L   A   L   E   K   F   E    880

2641  GAG GAG CGC ATC AGC CGT GGG GGG TCC CTC AAG GGG GAT GGA GGG GAC   2688
 881    E   E   R   I   S   R   G   G   S   L   K   G   D   G   G   D    896

2689  CGA TCC AGT GCC CTG GAC AAC CAG AGG ACC CCT TTG TCC CTG GGC CAG   2736
 897    R   S   S   A   L   D   N   Q   R   T   P   L   S   L   G   Q    912

2737  CGG GAG CCA CCA TGG CTG GCC AGG CCC TGT CAT GGA AAC TGT GAC CCG   2784
 913    R   E   P   P   W   L   A   R   P   C   H   G   N   C   D   P    928

2785  ACT CAG CAG GAG GCA GGG GGA GGA GAG TCT GTG GTG ACC TTT GAG GAC   2832
 929    T   Q   Q   E   A   G   G   G   E   S   V   V   T   F   E   D    944

2833  CGG GCC AGG CAC AGG CAG AGC CAA CGG CGC AGC CGG CAT CGC CGC GTC   2880
 945    R   A   R   H   R   Q   S   Q   R   R   S   R   H   R   R   V    960

2881  AGG ACA GAA GGC AAG GAG TCC TCT TCA GCC TCC CGG AGC AGG TCT GCC   2928
 961    R   T   E   G   K   E   S   S   S   A   S   R   S   R   S   A    976

2929  AGC CAG GAA CGC AGT CTG GAT GAA GCC ATG CCC ACT GAA GGG GAG AAG   2976
 977    S   Q   E   R   S   L   D   E   A   M   P   T   E   G   E   K    992

2977  GAC CAT GAG CTC AGG GGC AAC CAT GGT GCC AAG GAG CCA ACG ATC CAA   3024
 993    D   H   E   L   R   G   N   H   G   A   K   E   P   T   I   Q   1008
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Fig. 1 (4 con't)

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3025  GAA GAG AGA GCC CAG GAT TTA AGG AGG ACC AAC AGT CTG ATG GTG TCC   3072
1009   E   E   R   A   Q   D   L   R   R   T   N   S   L   M   V   S   1024

3073  AGA GGC TCC GGG CTG GCA GGA GGC CTT GAT GAG GCT GAC ACC CCC CTA   3120
1025   R   G   S   G   L   A   G   G   L   D   E   A   D   T   P   L   1040

3121  GTC CTG CCC CAT CCT GAG CTG GAA GTG GGG AAG CAC GTG GTG CTG ACG   3168
1041   V   L   P   H   P   E   L   E   V   G   K   H   V   V   L   T   1056

3169  GAG CAG GAG CCA GAA GGC AGC AGT GAG CAG GCC CTG CTG GGG AAT GTG   3216
1057   E   Q   E   P   E   G   S   S   E   Q   A   L   L   G   N   V   1072

3217  CAG CTA GAC ATG GGC CGC GTC ATC AGC CAG AGC GAG CCT GAC CTC TCC   3264
1073   Q   L   D   M   G   R   V   I   S   Q   S   E   P   D   L   S   1088

3265  TGC ATC ACG GCC AAC ACG GAC AAG GCC ACC ACC GAG AGC ACC AGC GTC   3312
1089   C   I   T   A   N   T   D   K   A   T   T   E   S   T   S   V   1104

3313  ACC GTC GCC ATC CCC GAC GTG GAC CCC TTG GTG GAC TCA ACC GTG GTG   3360
1105   T   V   A   I   P   D   V   D   P   L   V   D   S   T   V   V   1120

3361  CAC ATT AGC AAC AAG ACG GAT GGG GAA GCC AGT CCC TTG AAG GAG GCA   3408
1121   H   I   S   N   K   T   D   G   E   A   S   P   L   K   E   A   1136

3409  GAG ATC AGA GAG GAT GAG GAG GAG GTG GAG AAG AAG AAG CAG AAG AAG   3456
1137   E   I   R   E   D   E   E   E   V   E   K   K   K   Q   K   K   1152

3457  GAG AAG CGT GAG ACA GGC AAA GCC ATG GTG CCC CAC AGC TCA ATG TTC   3504
1153   E   K   R   E   T   G   K   A   M   V   P   H   S   S   M   F   1168

3505  ATC TTC AGC ACC ACC AAC CCG ATC CGG AGG GCC TGC CAC TAC ATC GTG   3552
1169   I   F   S   T   T   N   P   I   R   R   A   C   H   Y   I   V   1184

3553  AAC CTG CGC TAC TTT GAG ATG TGC ATC CTC CTG GTG ATT GCA GCC AGC   3600
1185   N   L   R   Y   F   E   M   C   I   L   L   V   I   A   A   S   1200

3601  AGC ATC GCC CTG GCG GCA GAG GAC CCC GTC CTG ACC AAC TCG GAG CGC   3648
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Fig. 1 (5 con't)

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1201   S   I   A   L   A   A   E   D   P   V   L   T   N   S   E   R     1216

3649 AAC AAA GTC CTG AGG TAT TTT GAC TAT GTG TTC ACG GGC GTG TTC ACC       3696
1217   N   K   V   L   R   Y   F   D   Y   V   F   T   G   V   F   T     1232

3697 TTT GAG ATG GTT ATA AAG ATG ATA GAC CAA GGC TTG ATC CTG CAG GAT       3744
1233   F   E   M   V   I   K   M   I   D   Q   G   L   I   L   Q   D     1248

3745 GGG TCC TAC TTC CGA GAC TTG TGG AAC ATC CTG GAC TTT GTG GTG GTC       3792
1249   G   S   Y   F   R   D   L   W   N   I   L   D   F   V   V   V     1264

3793 GTT GGC GCA TTG GTG GCC TTT GCT CTG GCG AAC GCT TTG GGA ACC AAC       3840
1265   V   G   A   L   V   A   F   A   L   A   N   A   L   G   T   N     1280

3841 AAA GGA CGG GAC ATC AAG ACC ATC AAG TCT CTG CGG GTG CTC CGA GTT       3888
1281   K   G   R   D   I   K   T   I   K   S   L   R   V   L   R   V     1296

3889 CTA AGG CCA CTG AAA ACC ATC AAG CGC TTG CCC AAG CTC AAG GCC GTC       3936
1297   L   R   P   L   K   T   I   K   R   L   P   K   L   K   A   V     1312

3937 TTC GAC TGC GTA GTG ACC TCC TTG AAG AAT GTC TTC AAC ATA CTC ATT       3984
1313   F   D   C   V   V   T   S   L   K   N   V   F   N   I   L   I     1328

3985 GTG TAC AAG CTC TTC ATG TTC ATC TTT GCT GTC ATC GCA GTT CAG CTC       4032
1329   V   Y   K   L   F   M   F   I   F   A   V   I   A   V   Q   L     1344


4033 TTC AAG GGA AAG TTC TTT TAT TGC ACG GAC AGT TCC AAG GAC ACA GAG       4080
1345   F   K   G   K   F   F   Y   C   T   D   S   S   K   D   T   E     1360

4081 AAG GAG TGC ATA GGC AAC TAT GTA GAT CAT GAG AAA AAC AAG ATG GAG       4128
1361   K   E   C   I   G   N   Y   V   D   H   E   K   N   K   M   E     1376

4129 GTG AAG GGC CGG GAA TGG AAG CGC CAT GAA TTC CAC TAC GAC AAC ATT       4176
1377   V   K   G   R   E   W   K   R   H   E   F   H   Y   D   N   I     1392

4177 ATC TGG GCC CTG CTG ACC CTC TTC ACC GTC TCC ACA GGG GAA GGA TGG       4224
1393   I   W   A   L   L   T   L   F   T   V   S   T   G   E   G   W     1408
```

Fig. 1 (6 con't)

```
4225  CCT CAA GTT CTG CAG CAC TCT GTA GAT GTG ACA GAG GAA GAC CGA GGC   4272
1409    P   Q   V   L   Q   H   S   V   D   V   T   E   E   D   R   G   1424

4273  CCA AGC CGC AGC AAC CGC ATG GAG ATG TCT ATC TTT TAT GTA GTC TAC   4320
1425    P   S   R   S   N   R   M   E   M   S   I   F   Y   V   V   Y   1440

4321  TTT GTG GTC TTC CCC TTC TTC TTT GTC AAT ATC TTT GTG GCT CTC ATC   4368
1441    F   V   V   F   P   F   F   F   V   N   I   F   V   A   L   I   1456

4369  ATC ATC ACC TTC CAG GAG CAA GGG GAT AAG ATG ATG GAG GAG TGC AGC   4416
1457    I   I   T   F   Q   E   Q   G   D   K   M   M   E   E   C   S   1472

4417  CTG GAG AAG AAT GAG AGG GCG TGC ATC GAC TTC GCA ATC AGC GCC CAA   4464
1473    L   E   K   N   E   R   A   C   I   D   F   A   I   S   A   Q   1488

4465  CCT CTC ACC CGC TAC ATG CCG CAG AAC AGA CAC ACC TTC CAG TAC CGC   4512
1489    P   L   T   R   Y   M   P   Q   N   R   H   T   F   Q   Y   R   1504

4513  GTG TGG CAC TTT GTG GTG TCT CCG TCC TTT GAG TAC ACC ATT ATG GCC   4560
1505    V   W   H   F   V   V   S   P   S   F   E   Y   T   I   M   A   1520

4561  ATG ATC GCC TTG AAT ACT GTT GTG CTG ATG ATG AAG TAT TAT TCT GCT   4608
1521    M   I   A   L   N   T   V   V   L   M   M   K   Y   Y   S   A   1536

4609  CCC TGT ACC TAT GAG CTG GCC CTG AAG TAC CTG AAT ATC GCC TTC ACC   4656
1537    P   C   T   Y   E   L   A   L   K   Y   L   N   I   A   F   T   1552

4657  ATG GTG TTT TCC CTG GAA TGT GTC CTG AAG GTC ATC GCT TTT GGC TTT   4704
1553    M   V   F   S   L   E   C   V   L   K   V   I   A   F   G   F   1568

4705  TTG AAC TAT TTC CGA GAC ACC TGG AAT ATC TTT GAC TTC ATC ACC GTG   4752
1569    L   N   Y   F   R   D   T   W   N   I   F   D   F   I   T   V   1584

4753  ATT GGC AGT ATC ACA GAA ATT ATC CTG ACA GAC AGC AAG CTG GTG AAC   4800
1585    I   G   S   I   T   E   I   I   L   T   D   S   K   L   V   N   1600

4801  ACC AGT GGC TTC AAT ATG AGC TTT CTG AAG CTC TTC CGA GCT GCC CGC   4848
1601    T   S   G   F   N   M   S   F   L   K   L   F   R   A   A   R   1616
```

Fig. 1 (7 con't)

```
4849  CTC ATA AAG CTC CTG CGT CAG GGC TAT ACC ATA CGC ATT TTG CTG TGG   4896
1617   L   I   K   L   L   R   Q   G   Y   T   I   R   I   L   L   W   1632

4897  ACC TTT GTG CAG TCC TTT AAG GCC CTC CCT TAT GTC TGC CTT TTA ATT   4944
1633   T   F   V   Q   S   F   K   A   L   P   Y   V   C   L   L   I   1648

4945  GCC ATG CTT TTC TTC ATT TAT GCC ATC ATT GGG ATG CAG GTA TTT GGA   4992
1649   A   M   L   F   F   I   Y   A   I   I   G   M   Q   V   F   G   1664

4993  AAC ATA AAA TTA GAC GAG GAG AGT CAC ATC AAC CGG CAC AAC AAC TTC   5040
1665   N   I   K   L   D   E   E   S   H   I   N   R   H   N   N   F   1680

5041  CGG AGT TTC TTT GGG TCC CTA ATG CTA CTC TTC AGG AGT GCC ACA GGT   5088
1681   R   S   F   F   G   S   L   M   L   L   F   R   S   A   T   G   1696

5089  GAG GCC TGG CAG GAG ATT ATG CTG TCA TGC CTT GGG GAG AAG GGC TGT   5136
1697   E   A   W   Q   E   I   M   L   S   C   L   G   E   K   G   C   1712

5137  GAG CCT GAC ACC ACC GCA CCA TCA GGG CAG AAC GAG AAC GAA CGC TGC   5184
1713   E   P   D   T   T   A   P   S   G   Q   N   E   N   E   R   C   1728

5185  GGC ACC GAT CTG GCC TAC GTG TAC TTT GTC TCC TTC ATC TTC TTC TGC   5232
1729   G   T   D   L   A   Y   V   Y   F   V   S   F   I   F   F   C   1744

5233  TCC TTC TTG ATG CTC AAC CTG TTT GTG GCC GTC ATC ATG GAC AAC TTT   5280
1745   S   F   L   M   L   N   L   F   V   A   V   I   M   D   N   F   1760

5281  GAG TAC CTG ACT CGG GAC TCC TCC ATC CTG GGG CCT CAC CAC TTG GAC   5328
1761   E   Y   L   T   R   D   S   S   I   L   G   P   H   H   L   D   1776

5329  GAG TTT GTC CGC GTC TGG GCA GAA TAT GAC CGA GCA GCA TGT GGC CGC   5376
1777   E   F   V   R   V   W   A   E   Y   D   R   A   A   C   G   R   1792

5377  ATC CAT TAC ACT GAG ATG TAT GAA ATG CTG ACT CTC ATG TCA CCT CCG   5424
1793   I   H   Y   T   E   M   Y   E   M   L   T   L   M   S   P   P   1808

5425  CTA GGC CTC GGC AAG AGA TGT CCC TCC AAA GTG GCA TAT AAG AGG TTG   5472
1809   L   G   L   G   K   R   C   P   S   K   V   A   Y   K   R   L   1824
```

Fig. 1 (8 con't)

```
5473  GTC CTG ATG AAC ATG CCA GTA GCT GAG GAC ATG ACG GTC CAC TTC ACC   5520
1825   V   L   M   N   M   P   V   A   E   D   M   T   V   H   F   T   1840

5521  TCC ACA CTT ATG GCT CTG ATC CGG ACA GCT CTG GAC ATT AAA ATT GCC   5568
1841   S   T   L   M   A   L   I   R   T   A   L   D   I   K   I   A   1856

5569  AAA GGT GGT GCA GAC AGG CAG CAG CTA GAC TCA GAG CTA CAA AAG GAG   5616
1857   K   G   G   A   D   R   Q   Q   L   D   S   E   L   Q   K   E   1872

5617  ACC CTA GCC ATC TGG CCT CAC CTA TCC CAG AAG ATG CTG GAT CTG CTT   5664
1873   T   L   A   I   W   P   H   L   S   Q   K   M   L   D   L   L   1888

5665  GTG CCC ATG CCC AAA GCC TCT GAC CTG ACT GTG GGC AAA ATC TAT GCA   5712
1889   V   P   M   P   K   A   S   D   L   T   V   G   K   I   Y   A   1904

5713  GCA ATG ATG ATC ATG GAC TAC TAT AAG CAG AGT AAG GTG AAG AAG CAG   5760
1905   A   M   M   I   M   D   Y   Y   K   Q   S   K   V   K   K   Q   1920

5761  AGG CAG CAG CTG GAG GAA CAG AAA AAT GCC CCC ATG TTC CAG CGC ATG   5808
1921   R   Q   Q   L   E   E   Q   K   N   A   P   M   F   Q   R   M   1936

5809  GAG CCT TCA TCT CTG CCT CAG GAG ATC ATT GCT AAT GCC AAA GCC CTG   5856
1937   E   P   S   S   L   P   Q   E   I   I   A   N   A   K   A   L   1952

5857  CCT TAC CTC CAG CAG GAC CCC GTT TCA GGC CTG AGT GGC CGG AGT GGA   5904
1953   P   Y   L   Q   Q   D   P   V   S   G   L   S   G   R   S   G   1968

5905  TAC CCT TCG ATG AGT CCA CTC TCT CCC CAG GAT ATA TTC CAG TTG GCT   5952
1969   Y   P   S   M   S   P   L   S   P   Q   D   I   F   Q   L   A   1984

5953  TGT ATG GAC CCC ACC GAT GAC GGA CAG TTC CAA GAA CGG CAG TCT CTG   6000
1985   C   M   D   P   T   D   D   G   Q   F   Q   E   R   Q   S   L   2000

6001  GTG GTG ACA GAC CCT AGC TCC ATG AGA CGT TCA TTT TCC ACT ATT CGG   6048
2001   V   V   T   D   P   S   S   M   R   R   S   F   S   T   I   R   2016

6049  GAT AAG CGT TCA AAT TCC TCG TGG TTG GAG GAA TTC TCC ATG GAG CGA   6096
2017   D   K   R   S   N   S   S   W   L   E   E   F   S   M   E   R   2032
```

Fig. 1 (9 con't)

```
6097  AGC AGT GAA AAT ACC TAC AAG TCC CGT CGC CGG AGT TAC CAC TCC TCC   6144
2033   S   S   E   N   T   Y   K   S   R   R   R   S   Y   H   S   S   2048

6145  TTG CGG CTG TCA GCC CAC CGC CTG AAC TCT GAT TCA GGC CAC AAG TCT   6192
2049   L   R   L   S   A   H   R   L   N   S   D   S   G   H   K   S   2064

6193  GAC ACT CAC CGC TCA GGG GGC AGG GAG CGG GGA CGA TCA AAA GAG CGA   6240
2065   D   T   H   R   S   G   G   R   E   R   G   R   S   K   E   R   2080

6241  AAG CAT CTT CTC TCT CCT GAT GTC TCC CGC TGC AAT TCA GAA GAG CGA   6288
2081   K   H   L   L   S   P   D   V   S   R   C   N   S   E   E   R   2096

6289  GGG ACC CAG GCT GAC TGG GAG TCC CCA GAG CGC CGT CAA TCC AGG TCA   6336
2097   G   T   Q   A   D   W   E   S   P   E   R   R   Q   S   R   S   2112

6337  CCC AGT GAG GGC AGG TCA CAG ACG CCC AAC AGA CAG GGC ACA GGT TCC   6384
2113   P   S   E   G   R   S   Q   T   P   N   R   Q   G   T   G   S   2128

6385  CTA AGT GAG AGC TCC ATC CCC TCT GTC TCT GAC ANC AGC ACC CCA AGA   6432
2129   L   S   E   S   S   I   P   S   V   S   D   X   S   T   P   R   2144

6433  AGA AGT CGT CGG CAG CTC CCA CCC GTC CCG CCA AAG CCC CGG CCC CTC   6480
2145   R   S   R   R   Q   L   P   P   V   P   P   K   P   R   P   L   2160

6481  CTT TCC TAC AGC TCC CTG ATT CGA CAC GCG GGC AGC ATC TCT CCA CCT   6528
2161   L   S   Y   S   S   L   I   R   H   A   G   S   I   S   P   P   2176

6529  GCT GAT GGA AGC GAG GAG GGC TCC CCG CTG ACC TCC CAA GCT CTG GAG   6576
2177   A   D   G   S   E   E   G   S   P   L   T   S   Q   A   L   E   2192

6577  AGC AAC AAT GCT TGC CTG ACC GAG TCT TCC AAC TCT CCG CAC CCC CAG   6624
2193   S   N   N   A   C   L   T   E   S   S   N   S   P   H   P   Q   2208

6625  CAG AGC CAA CAT GCC TCC CCA CAG CGC TAC ATC TCC GAG CCC TAC TTG   6672
2209   Q   S   Q   H   A   S   P   Q   R   Y   I   S   E   P   Y   L   2224

6673  GCC CTG CAC GAA GAC TCC CAC GCC TCA GAC TGT GGT GAG GAG GAG ACG   6720
2225   A   L   H   E   D   S   H   A   S   D   C   G   E   E   E   T   2240
```

Fig. 1 (10 con't)

```
6721  CTC ACT TTC GAA GCA GCC GTG GCT ACT AGC CTG GGC CGT TCC AAC ACC   6768
2241   L   T   F   E   A   A   V   A   T   S   L   G   R   S   N   T   2256

6769  ATC GGC TCA GCC CCA CCC CTG CGG CAT AGC TGG CAG ATG CCC AAC GGG   6816
2257   I   G   S   A   P   P   L   R   H   S   W   Q   M   P   N   G   2272

6817  CAC TAT CGG CGG CGG AGG CGC GGG GGG CCT GGG CCA GGC ATG ATG TGT   6864
2273   H   Y   R   R   R   R   R   G   G   P   G   P   G   M   M   C   2288

6865  GGG GCT GTC AAC AAC CTG CTA AGT GAC ACG GAA GAA GAT GAC AAA TGC   6912
2289   G   A   V   N   N   L   L   S   D   T   E   E   D   D   K   C   2304

6913  TAG AGG CAG C                                                   6922
2305   *   R   Q                                                       2307
```

Fig. 1 (11 con't)

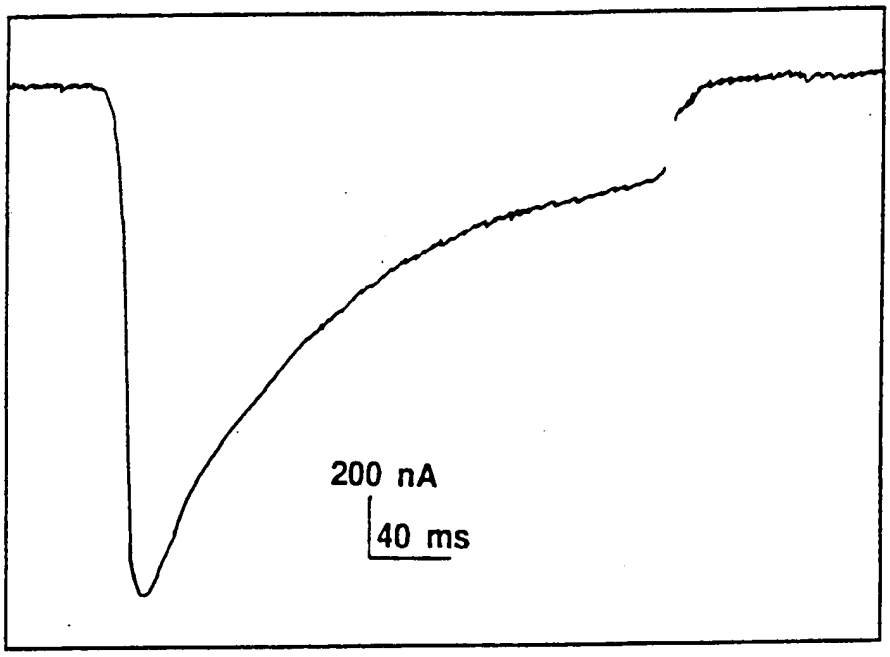

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08589

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12N 15/12, 15/63, 5/10; C12Q 1/02
US CL :536/23.5; 435/320.1, 240.2, 4; 530/350, 395
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5; 435/320.1, 240.2, 4; 530/350, 395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 260, issued 21 May 1993, T. W. Soong et al., "Structure and Functional Expression of a Member of the Low Voltage-Activated Calcium Channel Family", pages 1133-1136, especially the abstract, introduction, Fig. 1, and footnotes nos. 9 and 11.	1-10
Y	Genbank sequence database record, Accession no. L15453, issued 28 May 1993, see the entire record.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search
03 OCTOBER 1994

Date of mailing of the international search report
27 OCT 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks

Authorized officer
DAVID T. FITZGERALD

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Neuron, Volume 8, issued January 1992, M. E. Williams et al., "Structure and Functional Expression of α-1, α-2, and β Subunits of a Novel Human Neuronal Calcium Channel Subtype", pages 71-84, especially the abstract, Fig. 1, and the paragraph bridging pages 71-74.	1-10
Y	Science, Volume 231, issued 07 March 1986, N. Dascal et al., "Expression and Modulation of Voltage-Gated Calcium Channels After RNA Injection in Xenopus Oocytes", pages 1147-1150, especially the abstract, Figures 1 and 2, and the introduction.	4, 5, 7-10
A	EP, A2, 0,507,170 (FRANZ et al.) 07 October 1992; see especially Sequence No. 27980/2.	1-10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08589

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

USPTO-APS, Medline, Biosis, CAS, Derwent WPI
Search terms: calcium channel; alpha-1e; human; clon?, recombinant?, cDNA
IgSuite Sequence databases